APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2003, p. 5198–5206 Vol. 69, No. 9
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.9.5198–5206.2003

Effects of Soil pH on the Biodegradation of Chlorpyrifos and Isolation of a Chlorpyrifos-Degrading Bacterium

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Received 10 April 2003/Accepted 25 June 2003

We examined the role of microorganisms in the degradation of the organophosphate insecticide chlorpyrifos in soils from the United Kingdom and Australia. The kinetics of degradation in five United Kingdom soils varying in pH from 4.7 to 8.4 suggested that dissipation of chlorpyrifos was mediated by the cometabolic activities of the soil microorganisms. Repeated application of chlorpyrifos to these soils did not result in the development of a microbial population with an enhanced ability to degrade the pesticide. A robust bacterial population that utilized chlorpyrifos as a source of carbon was detected in an Australian soil. The enhanced ability to degrade chlorpyrifos in the Australian soil was successfully transferred to the five United Kingdom soils. Only soils with a pH of ≥6.7 were able to maintain this degrading ability 90 days after inoculation. Transfer and proliferation of degrading microorganisms from the Australian soil to the United Kingdom soils was monitored by molecular fingerprinting of bacterial 16S rRNA genes by PCR-denaturing gradient gel electrophoresis (DGGE). Two bands were found to be associated with enhanced degradation of chlorpyrifos. Band 1 had sequence similarity to enterics and their relatives, while band 2 had sequence similarity to strains of *Pseudomonas*. Liquid enrichment culture using the Australian soil as the source of the inoculum led to the isolation of a chlorpyrifos-degrading bacterium. This strain had a 16S rRNA gene with a sequence identical to that of band 1 in the DGGE profile of the Australian soil. DNA probing indicated that genes similar to known organophosphate-degrading (*opd*) genes were present in the United Kingdom soils. However, no DNA hybridization signal was detected for the Australian soil or the isolated degrader. This indicates that unrelated genes were present in both the Australian soil and the chlorpyrifos-degrading isolate. These results are consistent with our observations that degradation of chlorpyrifos in these systems was unusual, as it was growth linked and involved complete mineralization. As the 16S rRNA gene of the isolate matched a visible DGGE band from the Australian soil, the isolate is likely to be both prominent and involved in the degradation of chlorpyrifos in this soil.

Chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate] is used worldwide as an agricultural insecticide (4). Its environmental fate has been studied extensively, and the reported half-life in soil varies from 10 to 120 days (9, 18), with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. This large variation in half-life has been attributed to variation in factors such as pH, temperature, moisture content, organic carbon content, and pesticide formulation (2, 9). Initially, the high rate of chlorpyrifos degradation in soils with alkaline pH was attributed to chemical hydrolysis. Later, Racke et al. (20) concluded that the relationship between high soil pH and chemical hydrolysis was weak, since there was little degradation in several high-pH soils when sterile.

In general, pesticide degradation in soil can be influenced by both biotic and abiotic factors, which act in tandem and complement one another in the microenvironment. Environmental conditions play an important role in the survival and proliferation of microorganisms as well as affecting chemical stability. Chlorpyrifos is characterized by a P-O-C linkage as in other organophosphate pesticides, such as diazinon, parathion, methylparathion, and fenitrothion. Unlike in the case of other organophosphates, however, there have been no reports of enhanced degradation of chlorpyrifos since its first use in 1965. It has been suggested that the accumulation of TCP, which has antimicrobial properties, prevents the proliferation of chlorpyrifos-degrading microorganisms in soil (19). Robertson et al. (23) investigated chlorpyrifos behavior in a soil from a sugar cane field in Australia where the insecticide was no longer effective. They reported that chlorpyrifos was readily hydrolyzed to TCP in this alkaline soil. Transformation to TCP also occurred in fumigated soil, suggesting a nonmicrobial process. Fumigation of soil prevented rapid degradation of TCP, and they suggested that enhanced microbial degradation of TCP was involved in the loss of efficacy of chlorpyrifos in this particular soil.

Attempts to isolate chlorpyrifos-degrading bacteria from chlorpyrifos-treated soils have not been successful (13, 19). However, chlorpyrifos has been shown to be degraded cometabolically in liquid media by bacteria (10, 13), and various *opd* genes have been isolated from different microorganisms from different geographical regions, some of which have been shown to hydrolyze chlorpyrifos (10, 14, 22, 24). Several of the *opd* genes have similar DNA sequences (10, 14, 24) and can degrade a wide range of organophosphate compounds, while other genes are different from this group, although they have a similar organophosphate-hydrolyzing capability (29). The role of these *opd* genes in chlorpyrifos degradation in soil has not been studied.

The experiments reported here were carried out to investi-

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TABLE 1. Properties of the soils used in this study

Soil and sample	pH	% Organic matter	Moisture content (%)[a]	Microbial biomass (mg of C kg^{-1})	% Sand	% Silt	% Clay
UK soils							
1	4.7	2.33	15.9	150	86	8	6
2	5.7	2.67	16.8	172	83	10	7
3	6.7	2.79	16.7	189	84	9	7
4	7.7	3.02	17.7	201	76	10	14
5	8.4	3.12	17.9	200	85	8	7
Australian soil							
6	8.4	3.75	21.1	246	27	42	31

[a] 50% maximum water holding capacity.

gate the role of microorganisms in chlorpyrifos degradation in soils of different pHs, to determine the role of enhanced degradation in loss of efficacy of chlorpyrifos in an Australian soil, to examine the influence of soil pH on the survival of chlorpyrifos-degrading microorganisms, and to isolate a chlorpyrifos-mineralizing bacterium from the Australian soil.

MATERIALS AND METHODS

Pesticides and soils. Analytical-grade chlorpyrifos and TCP (99% purity; British Greyhound Ltd, Birkenhead, United Kingdom) were used throughout this study. Radiolabeled [2,6-ring-^{14}C]chlorpyrifos (946 MBq $mmol^{-1}$) was supplied by Dow Agroscience, Indianapolis, Ind.

Five soils with different pHs were collected from Deep Slade field at Horticulture Research International, Wellesbourne, United Kingdom. Triplicate soil samples were collected from the 0- to 10-cm layer. Separate trowels were used to collect each sample to avoid cross-contamination. Soil samples were partially air dried overnight and then sieved (3-mm mesh). A soil sample from a sugar cane field in the Burdekin River delta in Queensland, Australia, was supplied by Les Robertson, Commonwealth Scientific and Industrial Research Organisation. Chlorpyrifos had been used in this soil for more than 14 years, and it had recently failed to control target pests. The properties of the soils are listed in Table 1.

Chemical analysis. Details of the analytical methods for residues of chlorpyrifos and TCP were described previously (27). Soil samples were extracted with acetonitrile, and the extracts were analyzed by high-pressure liquid chromatography with appropriate standards to determine the levels of each compound. For the detection of radiolabeled compounds, subsamples (1 ml) of the acetonitrile extract were mixed with scintillation fluid (10 ml; Ecoscint A; National Diagnostics, Hessle, East Riding, Yorkshire, United Kingdom), and radioactivity was counted with a RackBeta liquid scintillation counter. Quench corrections were determined by external standard calibration. $^{14}CO_2$ from the soil samples was trapped in 5 ml of NaOH solution (1 M) in a sterile glass vial suspended within the soil incubation bottles. At intervals, the NaOH solution was recovered and diluted to 10 ml with distilled water, and 1-ml subsamples were mixed with 10 ml of Soluscint (National Diagnostics) and counted in the liquid scintillation counter.

Effects of soil pH on chlorpyrifos degradation in United Kingdom soils. Three replicate samples (100 g) of the United Kingdom soils were treated with a solution of 1 ml of [^{14}C]chlorpyrifos (83,600 Bq ml^{-1}) and 0.5 ml of technical chlorpyrifos (in methanol) to give 836 Bq g^{-1} and a chlorpyrifos concentration of 25 mg kg of dry $soil^{-1}$. Soil samples were left in a fume cupboard for 3 to 4 h for the solvent to evaporate, and distilled water was added to adjust the moisture contents to 40% of maximum water-holding capacity. Soils were mixed by hand and passed several times through a 3-mm mesh. Subsamples of 25 g of soil from each sample were transferred to sterile Duran bottles. A glass vial with 5 ml of NaOH (1 M) was placed in the bottle to trap $^{14}CO_2$. The remaining 75 g of soil from each treatment was transferred to sterile glass bottles and incubated at 20°C.

To study the effects of soil pH and repeated application on development of enhanced degradation of chlorpyrifos, three replicates (500 g) of the different pH soils from Deep Slade (Table 1) were treated with 2.5 ml of a solution of chlorpyrifos in methanol to give a concentration of 25 mg kg^{-1}. The soils were left on a laminar flow bench for 3 to 4 h to allow evaporation of the solvent, wetted to 40% of their water-holding capacity (Table 1), and incubated at 20°C. Samples were analyzed at regular intervals over the next 90 days. After 33 days of incubation, when half of the chlorpyrifos had dissipated from the three high-pH soils, 250 g of soil was recovered from each replicate and treated with chlorpyrifos again to achieve a final concentration of 25 mg kg^{-1} in each sample. Samples were mixed and the soils were handled as before. The soils were sampled periodically over 57 days. At 33 days after the second treatment, when half of the pesticide had again disappeared from high-pH soils, 100 g of soil was taken from each replicate and treated for a third time with chlorpyrifos to achieve 25 mg kg^{-1}. This time soils were sampled for a further 24 days. Moisture contents were maintained throughout the experiment by regular addition of sterile distilled water.

To examine the effects of change in soil pH on chlorpyrifos degradation, the two acidic soils (pH 4.7 and 5.7) were mixed with $CaCO_3$ at the rate of 10 g kg of $soil^{-1}$, to increase the pH (6). The pH was determined, chlorpyrifos was added to give a concentration of 25 mg kg^{-1}, and the soil samples were incubated as before.

Role of soil microorganisms in chlorpyrifos degradation. Subsamples (100 g) from the five soils of different pHs from Deep Slade were treated with chloroform (1 ml) and incubated in sealed jars for 7 days at 30°C. The chloroform was then removed by evacuation several times in a vacuum desiccator. Soil samples (100 g) were also treated with the antibacterial antibiotic chloramphenicol (2.5 ml, 4,800 mg $liter^{-1}$ in water) or the antifungal agent cycloheximide (2.5 ml; 4,800 mg $liter^{-1}$). The soils, including untreated controls, were thoroughly mixed and incubated at 20°C for 3 days. After pretreatment, chlorpyrifos was added as before at a concentration of 25 mg kg^{-1}. The samples were incubated at 20°C, and subsamples were taken at regular intervals for chemical analysis.

Effects of TCP on chlorpyrifos degradation. Further samples (100 g) of the different pH soils were treated with TCP to achieve concentrations of 25, 50, or 100 mg kg of $soil^{-1}$. After 7 days at 20°C, the samples were treated with chlorpyrifos at a concentration of 25 mg kg^{-1}. They were incubated at 20°C and sampled at regular intervals for 33 days.

Degradation of chlorpyrifos in the Australian soil. Triplicate samples of the Australian soil (Table 1) (250 g) were treated with [^{14}C]chlorpyrifos and technical chlorpyrifos (in 1 ml of methanol) to achieve a radioactivity of 836 Bq g^{-1} radioactivity and a concentration of 25 mg of chlorpyrifos kg^{-1}. After 33 days at 20°C, 150 g of soil from each replicate sample was retreated with radiolabeled and analytical chlorpyrifos to return the concentration to its initial value (836 Bq g^{-1} and 25 mg kg^{-1}). Soil samples were treated a third time 39 days after the first treatment.

To establish the role of microorganisms in degradation of chlorpyrifos in the Australian soil, triplicate samples were fumigated with chloroform or treated with the antibiotics cycloheximide, chloramphenicol, trimethoprim, and kanamycin as described previously. The effects of pretreatment of soil with TCP on degradation of chlorpyrifos were also studied in the Australian soil as described above.

Inoculation of United Kingdom soils with Australian soil. Samples of the five different United Kingdom soils (190 g) were mixed with 10 g of the Australian soil. Each sample was subsequently treated with ^{14}C and unlabeled chlorpyrifos to give concentrations of 836 Bq g^{-1} and 25 mg kg^{-1}. The soils were sampled periodically over the subsequent 40 days. This transfer procedure was repeated four times to examine the stability of the transferred degrading ability in the United Kingdom soils. On each occasion, 10 g of soil sample was mixed with 190 g of untreated United Kingdom soil. To further study the persistence of the microbial system responsible for enhanced degradation following mixing of the Australian soil into the five United Kingdom soils, a further experiment was carried out 90 days after preparation of the initial mixing experiment described above. All of the soil samples that had received a single dose of chlorpyrifos were retreated with labeled and unlabeled insecticide to give a radioactivity of 836 Bq g^{-1} and a concentration of 25 mg kg^{-1} soil. The soils were sampled at regular intervals over the subsequent 40 days to determine the rate of pesticide degra-

dation. At the end of the 40-day incubation period, the bacterial community structure was examined by PCR-denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene from total extractable DNA as described below.

16S rRNA profiling of soil bacterial populations. PCR amplification of the 16S rRNA gene followed by analysis of the products by DGGE was carried out to investigate changes in bacterial populations in soil. DNA was extracted from soil (1 g) using a commercial soil DNA clean kit (Mo Bio, Carlsbad, Calif.). PCR amplification of the 16S ribosomal DNA prior to DGGE was performed as described by Muyzer et al. (15). Thermocycling consisted of 35 cycles at 92°C for 45 s, 55°C for 30 s, and 68°C for 45 s, with 10 pmol of each of the primers. The primers amplified part of the eubacterial 16S rRNA gene corresponding to *Escherichia coli* nucleotide positions 341 to 534. PCR products (40 μl) were loaded onto an 8% (wt/vol) polyacrylamide gel and run in TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH 7.4). The polyacrylamide gels were made with a denaturing gradient from 40 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). The gel was run for 16 h at 60 V at 60°C (Bio-Rad, Richmond, Calif.). After electrophoresis, the gels were stained with ethidium bromide (0.5 mg l^{-1}) for 30 min and destained in water for 15 min.

The central portions of selected DGGE bands were excised with a sterile razor blade and soaked overnight in 50 μl of purified water (Milli-Ro, Bedford, Mass.). A subsample (5 μl) was used to reamplify the product using the conditions described for the initial PCR. The PCR product was purified using a QIAquick PCR purification kit (Qiagen Ltd, Crawley, West Sussex, United Kingdom). Cycle sequencing was performed using the BigDye *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Warrington, Cheshire, United Kingdom), and samples were analyzed on an ABI automated sequencer. The sequences obtained were edited by using SeqMan (Lasergene Inc., Madison, Wis.) and compared with database sequences by using the program MATCH for the Ribosomal Database Project II (http://rdp.cme.msu.edu/html) and FASTA 3 (http://www.ebi.ac.uk/embl/index.html) for the EMBL database.

Isolation of chlorpyrifos-degrading bacteria from the Australian soil. An attempt was made to isolate chlorpyrifos-degrading bacteria from the Australian soil following three successive treatments with chlorpyrifos and several rounds of enrichment in minimal salts medium supplemented with nitrogen (MSMN) with chlorpyrifos as the only source of carbon (5). The enrichment culture was serially diluted with MSMN, and aliquots were spread on MSMN agar plates. Approximately 200 colonies were randomly transferred from the solid MSMN and tested for their degrading ability by inoculation of liquid MSMN (5 ml in a universal bottle). Chlorpyrifos degradation was monitored for 8 weeks. Isolates positive for chlorpyrifos degradation (>20% removed) were streaked onto fresh chlorpyrifos-containing MSMN agar plates and to nutrient agar and Luria-Bertani plates to confirm purity. Pure isolates were characterized by sequencing of the 16S rRNA gene. Genomic DNA was isolated from bacterial colonies using a PrepMan sample preparation reagent (Applied Biosystems). The 16S rRNA gene was amplified by a set of primers which gave products of 500 bp. The forward primer began at base position 5 and the reverse primer began at base position 531. Amplification products were purified from excess primers and deoxynucleoside triphosphate by using Microcon 100 (Millipore, Watford, United Kingdom) molecular weight cutoff membranes.

Cycle sequencing of the 16S ribosomal DNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminator (Applied Biosystems). The same set of primers (5f and 531r) was used for sequencing packages. Samples were electrophoresed on ABI Prism 377 DNA sequencer (Applied Biosystems). Sequences were edited and identified as before.

Detection of *opd* in soils. The *E. coli* strain containing the cloned *Flavobacterium opd* gene was obtained from J. S. Karns, U.S. Department of Agriculture, Beltsville, Md. The strain was grown on either Luria broth or Luria agar containing 50 mg of ampicillin $liter^{-1}$. Three forward and three reverse primers were designed to conserved regions within database sequences of *opd* genes using information from gene alignments. Primers were obtained from Sigma-Aldrich (Dorset, United Kingdom). The six primers were *1f* (AAAGGCTGTGAGAG GATT), *2f* (GACGTCAGTTTATTGGCGA), *3f* (TGGTTCGACCCGCCAC TTTC), *1r* (TAGCTCGAAAACCCGAACAG), *2r* (CAAGAGAGCCCKTG TTTGC), and *3r* (CTTCTAGACCAATCGCACTG). DNA extracted from soil was serially diluted, and PCR amplification was attempted by using all possible paired combinations of forward and reverse primers. The PCR running conditions were the same as those for the 16S rRNA gene, except the annealing temperature was varied with a gradient from 52 to 60°C. DNA from the *E. coli* strain containing *opd* was used as a positive control. The efficiency of detection was tested by adding different cell densities of *E. coli* containing *opd* to a soil (from 1 to 10^4 cells g of $soil^{-1}$). DNA was extracted from the soil and used to amplify *opd* as before. Subsamples (10 μl) of the PCR were analyzed by agarose gel electrophoresis (1.5% agarose).

The *opd* gene was used as a probe for DNA hybridization studies. In a standard PCR, with the primers *1f* and *1r* and the *E. coli opd* gene as a DNA template, a digoxigenin (DIG)-dUTP nucleotide was incorporated into the product as described by the manufacturers (Boehringer, Ingelheim, Germany). The PCR product was purified with the QIAquick PCR clean-up kit. DNA (0.5 μg) from different soils, positive control DNA from *E. coli* containing *opd*, and negative control DNA from *E. coli* were denatured and transferred to a nitrocellulose membrane (Bio-Rad). The DNA was fixed to the membrane by UV treatment. The filter was prehybridized overnight at 65°C in 5× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) with 0.1% (wt/vol) *N*-lauroylsarcosine, 1% (wt/vol) SDS, and 1% (wt/vol) Boehringer blocking reagent. The DIG-labeled gene probe (50 μl) was denatured at 100°C for 5 min, chilled on ice for 10 min, added to 5 ml of fresh hybridization solution, and placed over the membrane. After overnight incubation at 65°C, the membrane was washed twice with 2× SSC for 5 min and two washes with 2× SSC–0.1% sodium dodecyl sulfate at 65°C. The membrane was then equilibrated in 100 ml of maleic acid buffer and probed with the anti-DIG antibody as described by the manufacturers. The intensity of the hybridization signal was measured with Image-Pro Plus software.

Nucleotide sequence accession numbers. The two PCR products sequenced in this work, bands 1 and 2, have been deposited in the EMBL database under accession numbers AJ564997 and AJ564998, respectively.

RESULTS

Effects of soil pH on chlorpyrifos degradation in United Kingdom soils. Degradation of chlorpyrifos in the two acidic soils was slow, especially in the soil with pH 4.7 (Fig. 1A), where the half-life was 256 days. Chlorpyrifos degradation at pH 5.7 (Fig. 1B) was somewhat faster, with the half-life being 58 days. Formation of the metabolite TCP was more pronounced at pH 5.7 than at pH 4.7. Total radioactivity extracted from the two soils over time was similar, as was the amount of $^{14}CO_2$ released. Chlorpyrifos degradation in the more neutral pH 6.7 soil (Fig. 1C) was quicker than in the two acidic soils, with a half-life of 35 days. More TCP was formed in this soil during incubation. However, total extractable radioactivity and percentage release of $^{14}CO_2$ was similar to that in the acidic soils. Chlorpyrifos degradation was rapid in the two alkaline soils (pH 7.7 and 8.4) (Fig. 1D and E), with a half-life of 16 days in both of them. The concentrations of TCP formed were also high (up to 10 mg kg^{-1}). As with the neutral-pH soil, there was little change in extractable radioactivity, and only small amounts of $^{14}CO_2$ were released during 90 days of incubation.

The rates of degradation of chlorpyrifos after repeated application to the five United Kingdom soils are summarized in Table 2. The degradation rate was again low in the two acidic soils, with half-lives of 224 and 58 days at pH 4.7 and 5.7, respectively. Repeated treatments with chlorpyrifos did not lead to a change in either the degradation rate or the kinetics of degradation in these soils. Chlorpyrifos degradation was more rapid in neutral-pH (6.7) and alkaline soils, but repeated treatment of these soils with chlorpyrifos again had no effect on either degradation rate or kinetics. Repeated treatment of the three higher-pH soils with chlorpyrifos led to accumulation of TCP.

Addition of lime to the pH 4.7 soil increased its pH to 7.5, and a similar addition to the pH 5.7 soil increased its pH to 8.6. The degradation of chlorpyrifos in these modified-pH soils was similar to that observed in the soils that were originally of high pH (data not shown).

Role of soil microorganisms in chlorpyrifos degradation in United Kingdom soils. There was no degradation of chlorpyrifos or formation of TCP in the two acidic soils or in the

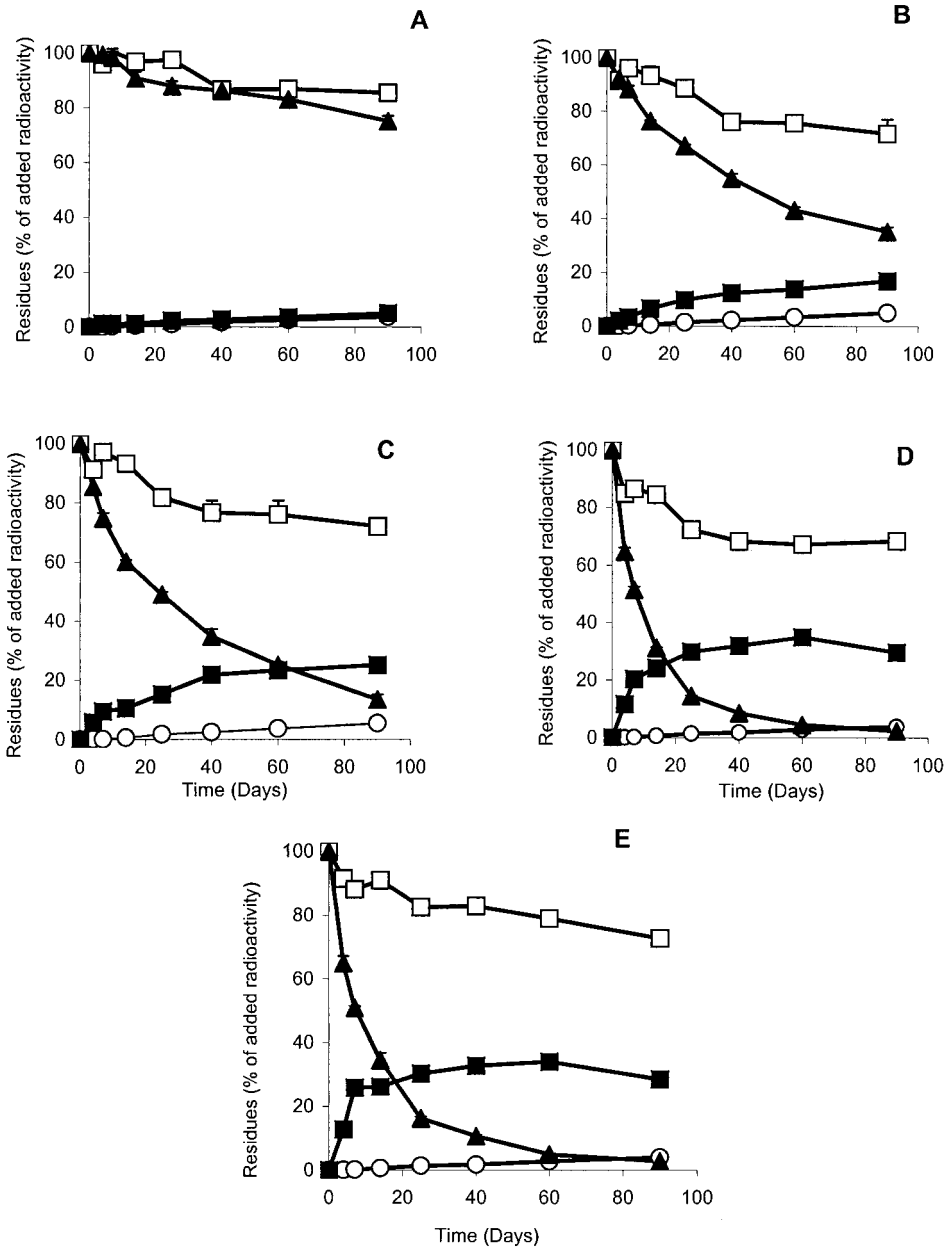

FIG. 1. Distribution of [^{14}C]chlorpyrifos and degradation products in five United Kingdom soils. (A) pH 4.7; (B) pH 5.7; (C) pH 6.7; (D) pH 7.7; (E) pH 8.4. ■, TCP; ▲, chlorpyrifos; □, radioactivity in soil; ○, $^{14}CO_2$. Standard errors are displayed ($n = 3$).

neutral-pH soil during the 33 days of incubation following fumigation with chloroform. There was only limited degradation under alkaline conditions, where less than 10% of applied chlorpyrifos was converted to TCP during 40 days of incubation. Effects of chloramphenicol on chlorpyrifos degradation were similar to those following fumigation with chloroform. Formation of TCP in alkaline soils was almost negligible. Cycloheximide had no effect on either chlorpyrifos degradation or formation of TCP in the soils.

Effect TCP on chlorpyrifos degradation in United Kingdom soils. There was no effect of TCP on the degradation of chlorpyrifos when it was added to soil at 25 mg kg^{-1} (Table 2). However, chlorpyrifos half-life increased as the pretreatment concentration of TCP increased. The degradation rate of TCP itself was also lower as the pretreatment concentration of TCP increased (Table 2).

Enhanced degradation of chlorpyrifos in an Australian soil. Chlorpyrifos degradation in the Australian soil following the first application of the insecticide indicated an initial lag phase followed by a period of rapid degradation (Fig. 2A). There was no lag phase following the second and third treatments (Fig. 2B and C). The pesticide was rapidly degraded and mineralized to $^{14}CO_2$. The half-life of chlorpyrifos in the first treatment was 15 days, and this was reduced to 3 and 2 days in the second and third treatments, respectively. Also, 64% of the applied radiolabeled pesticide was released as $^{14}CO_2$ in the

TABLE 2. Estimated first-order half-lives for degradation of chlorpyrifos and TCP in United Kingdom soils[a]

Treatment	Half-life (days) at soil pH[b]:				
	4.7	5.7	6.7	7.7	8.4
Repeated chlorpyrifos treatment					
First treatment	224 Aa	58 Ab	32 Ac	17 Ad	18 Ad
Second treatment	187 Ba	60 Ab	33 Ac	15 Ad	15 Ad
Third treatment	231 Aa	67 Ab	28 Ac	15 Ad	15 Ad
Pretreatment with TCP (mg/kg)					
25	257 Aa	64 Ab	26 Ac	13 Ad	13 Ad
50	385 Ba	107 Bb	41 Bc	28 Bd	40 Bd
100	578 Ca	154 Cb	62 Cc	40 Cd	36 Cd
Mixing with 5% Australian soil					
First treatment	15 Aa	8.5 Ab	6.4 Ac	6.7 Ac	6.4 Ac
Second treatment (after 90 days)	187 Ba	53 Bb	2.8 Bc	2.0 Bd	1.9 Bd
Effect of TCP concentration (mg/kg) on TCP degradation					
25	107 Aa	99 Aa	69 Ab	53 Abc	50 Ac
50	116 Aa	110 Aa	104 Ba	67 Ab	73 Bb
100	154 Ba	144 Aa	126 Ba	92 Bb	91 Bb

[a] Values were calculated from three replicate soil treatments as described by Singh et al. (27).
[b] Within any one of the four data sets, values followed by the same capital letter in the column or the same lowercase letter in the row are not significantly different ($P = 0.05$).

first treatment over the 46-day incubation period. Mineralization was greatly accelerated with the second and third treatments, and almost 80% of the applied pesticide was mineralized within 6 days in the third treatment. Initially, a small quantity of TCP was recovered from the soil, but this was degraded rapidly, and no TCP was recovered following the second and third treatments.

Fumigation of soil with chloroform gave complete inhibition of chlorpyrifos degradation. Small quantities of TCP were produced but did not exceed 5 to 10% of the applied insecticide.

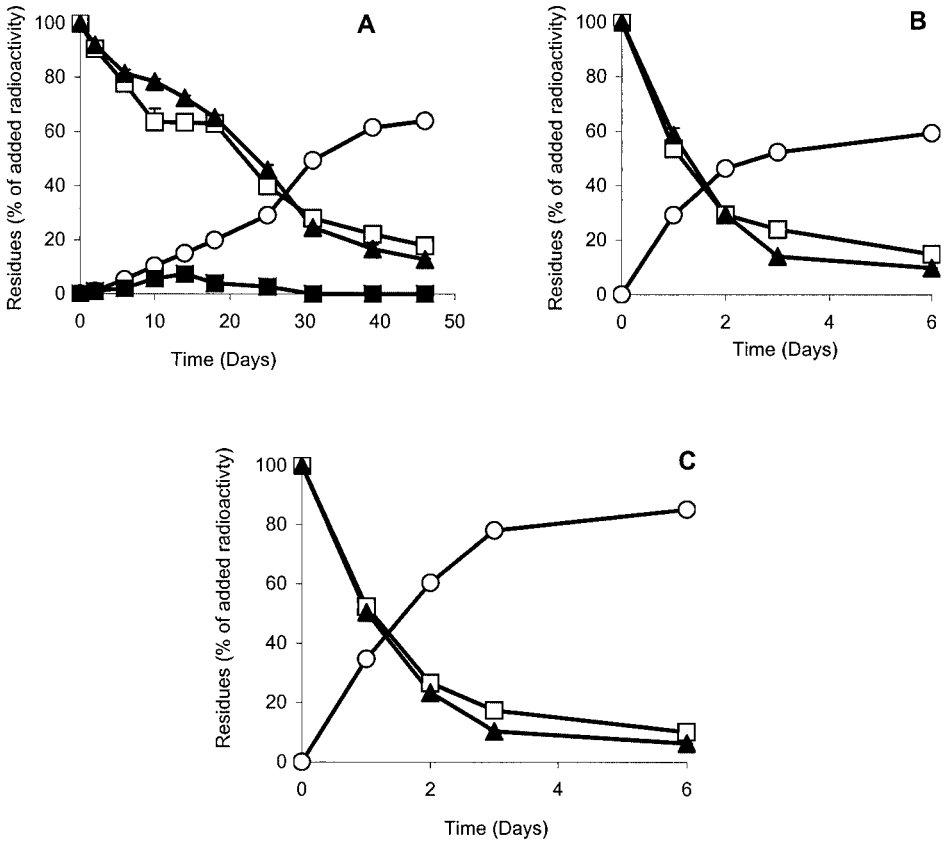

FIG. 2. Distribution of [^{14}C]chlorpyrifos and degradation products in the Australian soil. ■, TCP; ▲, chlorpyrifos; □, radioactivity in soil; ○, $^{14}CO_2$ after the first (A), second (B), and third (C) applications. Standard errors are displayed ($n = 3$).

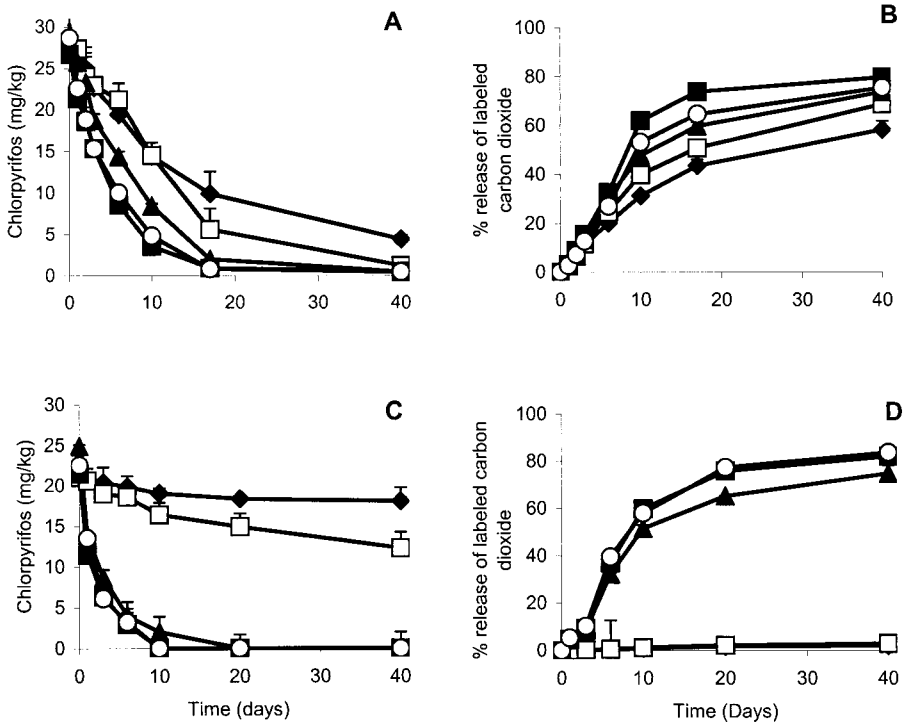

FIG. 3. Degradation of chlorpyrifos (A and C) and release of $^{14}CO_2$ (B and D) in United Kingdom soils immediately (A and B) and 90 days (C and D) after mixing with 5% of the enhanced Australian soil. Symbols: ◆, pH 4.7; □, pH 5.7; ▲, pH 6.7; ■, pH 7.7; ○, pH 8.4. Standard errors are displayed ($n = 3$).

The addition of chloramphenicol did not affect the degradation or mineralization of chlorpyrifos in the soil. However, the addition of trimethoprim and kanamycin reduced the degradation rate by 65%. The addition of cycloheximide also reduced the rate of pesticide mineralization, but the effect was relatively small (<15%). The combination of kanamycin and cycloheximide had a greater inhibitory effect (>85%) than kanamycin alone.

Prior treatment of soil with different concentrations of TCP had no effect on chlorpyrifos degradation. TCP itself was degraded rapidly in the Australian soil.

Inoculation of United Kingdom soils with the Australian soil. Degradation of chlorpyrifos in the United Kingdom Deep Slade soils inoculated with 5% of the enhanced Australian soil (Fig. 3A) was more rapid than in the soils with no addition. The first-order half-lives were 14.8 and 8.5 days at pH 4.7 and 5.7, respectively (Table 2). Degradation of chlorpyrifos in neutral-pH soil (6.7) mixed with 5% Australian soil gave a half-life of 6.4 days, and the amount of TCP formed was negligible. Degradation of chlorpyrifos was also rapid in the two alkaline soils when they were mixed with the Australian soil (Fig. 3A). The half-lives of chlorpyrifos for the first treatment were 6.7 and 6.4 days for soils at pH 7.7 and 8.4, respectively (Table 2). In all five soils, there was extensive mineralization of the pyridine ring (Fig. 3B), with up to 75% loss as $^{14}CO_2$.

The enhanced degrading ability of the two acidic soils was lost 90 days following the first mixing with Australian soil and in the absence of chlorpyrifos additions (Fig. 3C; Table 2). Repeated application of chlorpyrifos after this 90-day period failed to reinduce enhanced degradation in these soils. However, in the soil at pH 6.7 and in both of the alkaline soils, enhanced degrading capability was retained over this period, and the rate of degradation of chlorpyrifos remained high, with a half-life of just 2 to 3 days (Fig. 3B; Table 2). Mineralization of the pyridine ring also remained extensive in the three higher-pH soils (Fig. 3D), but negligible amounts of $^{14}CO_2$ were released from the acidic soils.

Profiling of bacterial 16S rRNA genes in soils. The effects of chlorpyrifos treatment on the bacterial community analyzed by PCR-DGGE in United Kingdom soils with different pH are shown in Fig. 4. Between 30 and 40 bands were present in each sample. The banding profiles indicate that there was little difference between the bacterial community structure in the different soils. It also shows that with the application of chlorpyrifos, there was little alteration of bacterial community structure in any of the soils. The PCR-DGGE analysis of DNA from the Australian soil and the United Kingdom soils mixed with 5% Australian soil (incubated for 90 days) is shown in Fig. 4 (lanes 13 to 18). Two main bands associated with the Australian soil were also detected in neutral-pH and alkaline United Kingdom soils inoculated with Australian soil but not in the inoculated acidic United Kingdom soils. Both bands were excised and sequenced. The sequence from band 1 showed similarity to 16S rRNA gene sequences from bacteria in the Ribosomal Database Project group that includes enteric organisms and their relatives, within the *Salmonella enterica* serovar Enteritidis, *Citrobacter*, *Pantoea*, and *Enterobacter* subgroups. The sequence had 96% similarity to *Pantoea stewartii* (accession no. PS296080) and *S. enterica* (accession no. AL627279). Band 2 was placed in the *Pseudomonas stutzeri*,

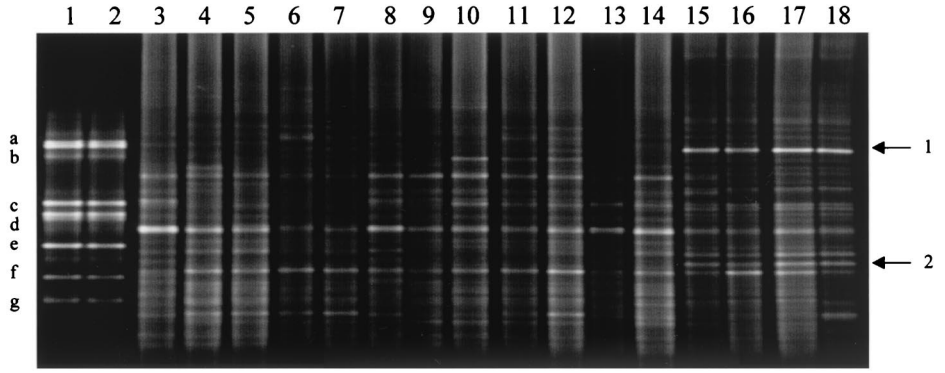

FIG. 4. PCR-DGGE analysis of bacterial communities in control, treated, and mixed United Kingdom and Australian soils. Lanes: 1 and 2, markers; 3 to 7, untreated United Kingdom soil; 8 to 12, United Kingdom soil treated with chlorpyrifos; 13 to 17, treated United Kingdom and Australian soil (mixed); 18, treated Australian soil. United Kingdom soils are arranged from pH 4.7 to 8.4. The marker consisted of *Pseudomonas fluorescens* (a), *Sphingomonas yanoikuyae* (b), *Bacillus subtilis* (c), *Burkholderia phenazinium* (d), *Paenibacillus amylolyticus* (e), *Agrobacterium rhizogenes* (f), and *Arthrobacter polychromogenes* (g). Two bands from the Australian soil that are present in the higher-pH United Kingdom soils 90 days after mixing are indicated with arrows.

Pseudomonas putida, and *Pseudomonas mendocina* subgroups of the group including *Pseudomonas* and relatives. The greatest similarity (100%) found was to *Pseudomonas putida* (accession no. X93997), *Pseudomonas stutzeri* (accession no. PST270451), and other related strains. As only a short DNA sequence was available from the PCR product (150 bp), further positioning of the sequences was not possible.

Isolation of chlorpyrifos-degrading bacteria from the Australian soil. Four positive isolates that utilized chlorpyrifos as a sole source of carbon were obtained from MSMN. Biochemical and molecular analyses confirmed that all isolates belonged to the same microbial type. To compare the isolated strain to the DGGE bands obtained from the soil, the same region of the 16S rRNA gene was sequenced. The sequence from the isolated strain was identical to the band 1 sequence from the Australian soil. The isolates were also found to be resistant to chloramphenicol.

Detection of *opd* in soils and isolated cultures. Attempts to amplify the *opd* gene from DNA extracted from soil using three sets of primers designed from the known *opd* genes were unsuccessful. DNA amplification only occurred with positive control DNA. Different dilutions of DNA and different annealing temperatures did not result in any amplification products for the samples. Soil spiked with small amounts of bacterial control DNA indicated that more than 100 cells per g of soil were required to provide positive amplification of the *opd* gene with the method used.

The results of direct hybridization of soil DNA using the *opd* probe are presented in Fig. 5. Strong signals were obtained from the positive control DNA and from DNA isolated from the alkaline United Kingdom soils, with only weak signals from acidic soils. No signal was detected from *E. coli* negative control DNA or from DNA extracted from the Australian soil. No PCR amplification product could be obtained from the isolated chlorpyrifos-degrading strain by using the primers designed for the *opd* gene. In addition, no hybridization signal was detected from the DNA isolated from this bacterium.

DISCUSSION

Many other organophosphate pesticides are susceptible to enhanced degradation in soil such as isofenophos (3, 17), fenamiphos (16), ethoprophos (12), diazinon (8), fensulfothion (21), parathion, and methylparathion (25), but there have been no reports of enhanced degradation of chlorpyrifos per se. There have, however, been reports of different rates of degradation of chlorpyrifos in soils, with a half-life range from 10 to 120 days (18). In the present experiments, the rate of degradation of chlorpyrifos was low in acidic soils but increased considerably with an increase in soil pH. There was no difference between soils in release of $^{14}CO_2$ from the pyridine ring, despite the large differences in degradation rate, indicating that the ring structure was not mineralized. Our results agree with the conclusions of Racke et al. (20) that the relationship

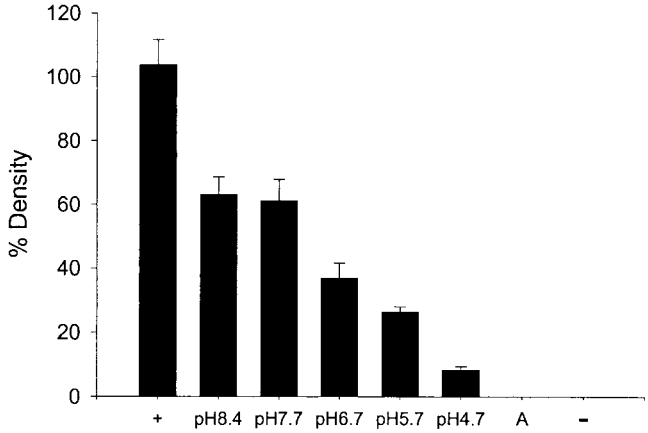

FIG. 5. Level of hybridization signal using an *opd* gene probe and target DNA extracted from the different soils. The intensity of color development was determined and is presented as a percentage (where 100% represents the signal obtained from *E. coli* cells containing the *opd* gene and 0% is the signal from *E. coli* cells). Standard errors are displayed ($n = 3$). +, positive control; −, negative control; A, Australian soil.

between high pH and rapid abiotic hydrolysis is poor, since high-pH soils failed to hydrolyze chlorpyrifos when sterilized, suggesting the involvement of soil microorganisms. Further experiments with antibiotics indicated a role of the bacterial community, since the antibacterial agent chloramphenicol inhibited degradation, whereas the antifungal agent cycloheximide had little effect.

Chlorpyrifos has been reported previously to be resistant to enhanced degradation, due to the accumulation in soil of the antimicrobial degradation product TCP. In the present experiments, an increase in pretreatment concentration of TCP in the soil reduced the degradation rate of chlorpyrifos, although concentrations greater than 25 mg kg^{-1} were required before this occurred. An increase in TCP concentration also reduced the degradation rate of TCP itself, which supports the earlier report of a possible toxic effect of this compound on the soil microbial community (19). However, when chlorpyrifos is used repeatedly over many years, some opportunist microorganisms may well develop the capability to use this toxic compound, as has been reported with organochlorine compounds (26). This adaptation can provide strains with a competitive advantage over other microbes in terms of sources of energy. This may well have occurred in the Australian soil, where chlorpyrifos had been used continuously for more than 14 years. This soil degraded both the parent compound and the degradation product TCP very rapidly, and the adapted microbial community was able to mineralize the pyridine ring. To the best of our knowledge, the present results provide the first report of enhanced microbial degradation of chlorpyrifos, although enhanced degradation of TCP has been reported before (23).

Complete mineralization of chlorpyrifos in the Australian soil appeared to result from the activities of both fungal and bacterial components of the soil microflora. Both antifungal and antibacterial agents affected the rate of degradation. The results from the mineralization experiment suggested that soil bacteria mainly brought about chlorpyrifos degradation. Chloramphenicol had no effect on mineralization rate, but the presence of kanamycin and trimethoprim in soil had a strong adverse effect, suggesting that chloramphenicol-resistant bacteria were responsible for degradation. Pretreatment of soil with TCP up to 100 mg kg^{-1} had no effect on the rate of degradation of chlorpyrifos in the enhanced Australian soil, suggesting that a robust chlorpyrifos-degrading population had developed.

DGGE analysis indicated only slight differences in soil microbial community structure in soils of different pHs. This is not unexpected, as all the soils came from the same field at Horticulture Research International, where the pH was found to vary naturally between different locations. The application of chlorpyrifos to the previously untreated soils had little effect on soil community structure. However, the major observation from the DGGE profile was that 90 days after 5% enhanced Australian soil was mixed into United Kingdom soils varying in pH, only neutral and alkaline soils were able to maintain strong bands that were initially associated with the Australian soil. These bands did not persist in the acidic soils. This visual result was strongly supported by the degradation data. In the incubation experiment carried out 90 days after the initial mixing, only neutral and alkaline pH soils were still able to degrade chlorpyrifos rapidly (Table 2), and only these soils retained the capacity to mineralize the pyridine ring to $^{14}CO_2$ (Fig. 3D). The studies on the survival of chlorpyrifos-degrading microorganisms in United Kingdom soils with different pHs are important, as they examine the ecological characteristics and physiological requirements of degrading bacteria.

There have been previous reports of nonspecific relationships between high pH and rapid biodegradation of triazine (11) and substituted urea (28) herbicides. Recently, Bending et al. (1) showed that pH-mediated spatial variability in isoproturon degradation across a field was linked to the distribution of pesticide-degrading *Sphingomonas* spp. Our results suggest that high pH not only is helpful in developing enhanced degradation but also is required to maintain this capability for long periods. The above observation was supported by sequencing two visible bands from the mixed soil which were prominent within the banding profile, one of which we later matched to a degrading isolate. In this regard, it is most likely that we were able to transfer two strains to the United Kingdom soil that were able to play a part in the degradation of chlorpyrifos. Sequence analysis of the two bands indicated that two different strains were present. These strains were similar to members of the enterics or pseudomonads, both of which are known to be metabolically diverse. During isolation we were able to obtain only one of these strains in a pure culture that maintained its degradative ability. There could be several reasons why we failed to isolate the second strain, including an inability to provide the correct culture conditions, instability of the degradative phenotype, or the possibility that the strain may degrade only breakdown products of chlorpyrifos (not tested). The isolate we did obtain from the Australian soil utilized chlorpyrifos as the sole source of carbon in liquid culture. To our knowledge, this is the first report of the isolation of a chlorpyrifos-degrading bacterium from an enhanced degrading soil, although a TCP-mineralizing bacterium has been isolated previously (7). However, cometabolic degradation of chlorpyrifos by bacteria isolated from enrichment cultures for related organophosphate compounds has been reported before (10, 13). The work also indicates that this isolate was likely to be involved in chlorpyrifos degradation in the soil itself, as it was a prominent band on the DGGE gel when the bacterial component of the soil was analyzed. In addition, the isolated bacterium was resistant to chloramphenicol, as was a component of the degradative microbial population present in the Australian soil.

PCR amplification and DNA hybridization studies were carried out to determine if genes similar to known *opd* genes were present in the different soils and the isolate. No amplification products were detected, although a hybridization signal was detected in alkaline United Kingdom soils. This indicated that similar but not identical genes were present in the United Kingdom soil, which may be involved in the cometabolic degradation of chlorpyrifos. No hybridization signal or PCR product was obtained in the Australian soil or the degrading isolate, which indicates that a gene(s) different from those identified previously was involved in chlorpyrifos degradation in these systems. This finding is supported by our observations that chlorpyrifos was used as a source of energy in the Australian soil and that the molecule was extensively mineralized. Most *opd*-mediated degradation in soil has previously been reported

to be cometabolic (11, 14), with only partial breakdown detected

In conclusion, the degradation rate of chlorpyrifos is strongly related to soil pH, and degradation is microbial and not due to abiotic hydrolysis. In the Australian soil, rapid loss of chlorpyrifos is due to enhanced degradation of the parent compound and not chemical hydrolysis to TCP. In the Australian soil and cultures of the chlorpyrifos-degrading bacterium isolated from this soil, complete degradation was shown to occur, and this involves genes that are different from those reported previously. Soil pH has also been shown to be important for maintaining the enhanced degrading capability of soil for prolonged periods. Characterizing the pathway for degradation and identifying the genes and enzymes involved in this process represent areas for further investigation.

ACKNOWLEDGMENT

This work was funded in part by the United Kingdom Biotechnology and Biological Sciences Research Council (BBSRC).

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